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Conservation of *nif*- and species-specific domains within repeated promoter sequences from fast-growing *Rhizobium* species

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ABSTRACT

In the fast-growing *Rhizobium* species, repeated DNA sequences, which include the promoter region of the *nif* HDK operon have been described. These repeated sequences are promoters which specifically activate transcription in the endosymbiotic state. Hybridization analysis of these sequences from *R. trifolii* has revealed that they may be involved in the species-specific activation of the various genes whose transcription they promote. Comparative analysis of various copies of these repeated sequences, from *R. trifolii* (the clover symbiont) and *R. meliloti* (the alfalfa symbiont), reveals the presence of domains of intra- and interspecific conservation within the promoter regions. We suggest that these promoter elements represent sites which are involved in the species-specific and general, *nif*-specific activation of *Rhizobium* symbiotic genes.

INTRODUCTION

The host-specific interactions of the *Rhizobium*-legume symbiosis have been studied extensively. Whilst most studies of host specificity have been concerned with the initial interaction of the symbionts (nodulation), the molecular basis of such interactions has not been defined. However, Vincent (1) noted that: "specificity becomes even more apparent when effectiveness of nitrogen fixation, and not merely nodule-forming ability is considered".

A specific class of *Rhizobium* repeated promoters, which activate transcription in the endosymbiotic state, has been described. *R. meliloti* repeated promoters have been characterized at the DNA sequence level (2). These promoter sequences were shown by S1 nuclease-protection experiments to activate gene expression only in the endosymbiotic state (2). Hybridization analysis indicated that these promoters were conserved in various fast-growing *Rhizobium* species.

Similar repeated DNA sequences have been characterized in *R. trifolii* (3). DNA sequence analysis indicated that these repeats constitute a family of promoters similar to those described in *R. meliloti*. However, hybridization analysis of the *R. trifolii* repeated promoter sequences demonstrated

that they were species specific. Accordingly, we proposed a model which accounts for the available genetic data relating to the species specificity of nitrogen fixation (3). According to this model, the appropriate species-specific, repeated promoter sequence is required for expression of the *nif*HDK genes (and other repeated promoter-activated symbiotic genes) by the appropriate fast-growing *Rhizobium* species which effectively nodulates a given host plant.

This paper addresses the molecular basis of host-specific interactions that occur in the later stages of the symbiosis and which appear to be specifically regulated via the *Rhizobium* repeated promoter sequences. Intra- and inter-species comparisons reveal conserved domains within these promoter regions which may be involved in general, *nif*-specific as well as species-specific activation of symbiotic gene expression. The results derived in this paper are consistent with the model for species-specific activation of symbiotic genes and also with hitherto unexplained molecular data obtained from the analysis of other *nif* gene promoters.

RESULTS

To facilitate the analysis of the repeated promoter sequences of *Rhizobium* the Align computer program (4), based on the Needleman and Wunsch algorithm (5), was used. Numerically-based comparative analyses remove possible operator bias.

Comparison of the *R. trifolii* and *R. meliloti* repeated sequences

Previously, repeated promoter sequences of either *R. trifolii* (3) or *R meliloti* (2) have been compared. However, the ability to perform both intra- and inter-species comparisons has only become possible with the availability of data from both species. The data sets used include the *R. trifolii* repeated sequence (RtRS) copies designated RtRS1, 2 and 3 (3). The RtRS1 sequence is the *nif*HDK promoter region of *R. trifolii* strain ANU843. The sequence of the *nif*HDK promoter region of *R. trifolii* strain SU329 was also used (6). The four *R. meliloti* sequences analysed are designated RmP1, P2, P3 and P4 (2). The RmP1 sequence is the *nif*HDK promoter region of *R. meliloti* strain 102F34.

The definitions used for designating a particular nucleotide as either *nif*-specific (N or n) or species-specific (S or s) are given in Figure 1. This figure shows the alignment of the eight sequences analysed in this study along with their nucleotide classification using the above system. Figure 2 is a diagramatic representation of the classification of the

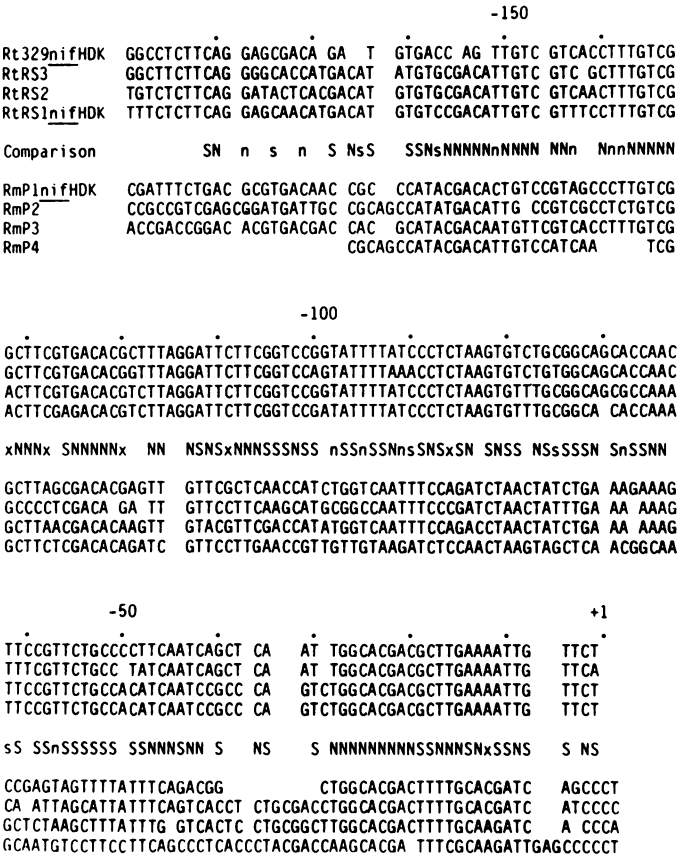

Figure 1: Comparison of the *R. trifolii* and *R. meliloti* repeated promoter sequences. The sequences were computer aligned (4) to determine homologous residues. Sequences used are *R. trifolii* SU329 *nif*H (6), *R. trifolii* repeated sequence RtRS1, 2 and 3 (3) and *R. meliloti* repeated sequences RmP1, P2, P3 and P4 (2). The results of comparisons of nucleotides at specific positions are classified as follows:

N (*nif*-specific) at least seven of the eight residues at that position are conserved in both species.

n (*nif*-specific) at least six of the eight residues at that position are conserved in both species.

S (species-specific) at least three of the four bases of one species are different from all of those of the second species.

s (species-specific) at least three of the four bases of one species are different from at least three of those of the second species.

If all four bases are conserved within one species and the second species has two pairs of differing residues, these nucleotides are also defined as species specific. However, if one of these pairs is the same as the four residues of the first species, that particular base could be defined as either species- or *nif*-specific. In such instances the nucleotide position is indicated by an x.

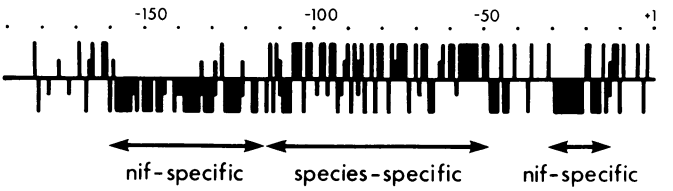

Figure 2: Diagramatic representation of the nucleotide positions as determined and classified in Fig. 1. Based on the specificity of nucleotide positions, *nif*-specific bases are indicated by large (N) and small (n) bars below the axis whilst species-specific bases are indicated by large (S) and small (s) bars above the axis. Nucleotides designated either species- or *nif*-specific (x) in Fig. 1 are indicated by small bars both above and below the axis. Domains of *nif*- and species-specific nucleotides are indicated.

nucleotides made in Figure 1.

Based on this analysis, the *Rhizobium* repeated promoter sequences contain three domains which may be involved in the control of symbiotic gene expression. The first of these domains is *nif*-specific and is located within the first 25 bp of the *Rhizobium* repeated promoter sequences. The sequences present in this region have previously been shown to be conserved in the promoter regions of various nitrogen fixation genes (7). Two regions have been identified, centred at the -10 and -20 positions, by analysis of *Klebsiella pneumoniae nif* promoters (8). The -10 region has been suggested to be formally analogous to the Pribnow box of *E. coli* (9), whilst the -20 region has been suggested to be the site of specific activation by the positive regulator of *nif* gene expression, the *nif*A gene product (8). Both the -10 and -20 regions have been identified in a number of comparisons of *nif* gene promoters (8,10-12) and are collectively referred to as the consensus promoter, as defined in *E. coli* (9).

The novel finding of this analysis is that two other extensive regions are present in the *Rhizobium* repeated promoters that contain domains of *nif*-specific and species-specific sequences. The species-specific sequences are located approximately between positions -118 and -45 (Figure 1). Within these 63 nucleotides, 38 (60%) are species-specific and 19 (30%) are *nif*-specific. However, given the random probability of any one position containing an identical nucleotide, the percentage of species-specific nucleotides may be as high as 80%.

A region of *nif*-specific nucleotides is located approximately between positions -165 and -122 and of the 44 bp located in this domain, 33 (75%) are *nif* specific whilst only two (5%) are species specific. Beyond position

Figure 3: Comparison of *Rhizobium* and *K. pneumoniae nif* promoters. The sequences used are the *K. pneumoniae nif*HDK (13) and *nif*LA (15) promoters. These sequences were computer aligned with the previous comparison of *R. trifolii* and *R. meliloti nif*HDK promoters. The positions of *nif*- (N,n) and species- (S,s) specific nucleotides determined in Fig. 1 are indicated. *K. pneumoniae nif*HDK or *nif*LA sequences that are homologous with *Rhizobium nif*-specific nucleotides are indicated in bold type face. Point mutations and a deletion that remove *nif*-inhibitory functions of the *K. pneumoniae nif*HDK promoter (18) are indicated above the *K. pneumoniae nif*HDK promoter sequence, the latter by overscoring.

-170 there is no significant intra- or interspecific homology between the various *Rhizobium* promoter sequences used in this study.

Conservation of *nif*-specific sequences in nitrogen-fixing organisms

The presence of *nif*-specific domains in *Rhizobium* repeated promoters suggests that these domains may be present in the *nif* promoters of other nitrogen-fixing organisms. Comparison of the *R. trifolii* and *R. meliloti nif*HDK promoter sequences with those of the *K. pneumoniae nif*HDK (13,14) and *nif*LA (15) genes was undertaken. A comparison of these sequences is presented in Figure 3. The *K. pneumoniae nif*HDK promoter sequence is 39% homologous with the *R. trifolii nif*HDK promoter and of these conserved bases 57% are also *nif* specific, as previously determined by the analysis of the *R. trifolii* and *R. meliloti* sequences. Similarly, alignment of the *K.*

*pneumoniae nif*HDK promoter with the *R. meliloti nif*HDK promoter reveals 34% overall homology and 60% of these conserved nucleotides are *nif* specific. When the *K. pneumoniae nif*LA promoter is aligned with either of the *Rhizobium nif*HDK promoters, 40% overall sequence homology is detected. As seen in the analysis of the *nif*HDK promoters, 56% of the conserved nucleotides are also *nif* specific. Comparison of the *K. pneumoniae nif*HDK and *nif*LA promoters also serves to confirm the regions of homology that are located upstream of the consensus -10 and -20 promoter elements. As seen in Figure 3, there are 70 nucleotides in both the *K. pneumoniae nif*HDK and *nif*LA sequences that are homologous with previously-identified *nif*-specific nucleotides. Of these 70 nucleotides, 41 (59%) are located in regions corresponding to the two *nif*-specific domains of the *Rhizobium* repeated promoters. The clustering of the *Rhizobium nif*-specific nucleotides and the homology of the *K. pneumoniae* sequences within these domains suggests that these *K. pneumoniae* sequences are also involved in *nif*-specific regulation.

Functional domains in *K. pneumoniae nif* promoters

Directed mutagenesis of the *Rhizobium* repeated promoters should facilitate an understanding of the roles of these sequences in the control of *nif* and species- or host-specific control of expression of the symbiotic genes. However, studies of the *K. pneumoniae nif*HDK (14) and *nif*LA (15) promoters do provide considerable evidence supporting the suggestion that domains of the *nif* promoter, other than the -10 and -20 consensus sequences, are involved in *nif*-specific regulation.

The *K. pneumoniae nif* promoters have been shown to inhibit nitrogen fixation when present on multicopy plasmids (16,17). This has been interpreted as multicopy promoter titration of the cellular pool of a positively-acting regulatory molecule (the *nif*A gene product, the *ntr*A gene product, or both) (16,17).

Based on these observations, mutant derivatives of multicopy plasmids, containing the *K. pneumoniae nif*HDK promoter, which overcome the inhibitory effect on nitrogen fixation were examined (18). Mutations that removed the inhibition of nitrogen fixation were shown to involve three separate single-base changes to the -10 region of the *Rhizobium* consensus promoter. The positions and nucleotide changes of these mutations are indicated in Figure 3. The other two mutations that relieved the inhibition of nitrogen fixation were a deletion from position -72 to -184 and a single base change at position -136 (indicated in Figure 3). The significance of these upstream mutations with respect to *nif*HDK transcription was not clear

(18). Based on the analysis presented in this paper, we suggest that the upstream mutations observed in *K. pneumoniae* disrupt a binding site for a regulatory protein. The upstream domain of *nif*-specific nucleotides is spanned by the *K. pneumoniae* deletion, and the single base change, from G to T, at position -136 is centred in a run of *nif*-specific residues. The correlation of this single base change with the Nif$^-$ phenotype and the upstream *nif*-specific domain of the *nif* promoters strongly suggests that this domain is involved in regulation of the nitrogen-fixing phenotype.

Analysis of the *K. pneumoniae nif*LA promoter has been undertaken by studying various deletion derivatives and their response to positive activation by the *ntr*C and *nif*A gene products (7,14,15,19,20). The response of the *nif*LA operon to the *ntr*C and *nif*A gene products is at the transcriptional level. Deletion analysis of the *K. pneumoniae nif*LA promoter revealed two unique aspects (15). Both promoter function and positive control by the *ntr*C and *nif*A gene products are retained in deletions extending as far as position -33, although transcriptional activity is reduced to only 20% of wild-type levels. Equally as significant, sequences as far as 150 bp upstream from the transcription initiation site are required for maximum promoter activity.

Both the *ntr*C and *nif*A gene products are biochemically (15,20) and structurally (21) similar and both require *ntr*A gene function for their regulatory activity (15,19,20,22). Indeed, the *nif*A product can substitute for the *ntr*C product in transcriptional activation of various genes involved in nitrogen metabolism (20,22). Due to the similarities of these two gene products, Drummond *et al.* (15) suggested that *ntr*C- or *nif*A-regulated promoters may show sequence homology. Some regions involved in this regulation are located in the first 30 bp of the promoter (15) but for maximal expression (*cf* 20% in the deletion derivatives) the gene requires the entire promoter of *ca* 150 bp.

The upstream *nif*-specific region identified in both *Rhizobium* and *K. pneumoniae* may be involved in the coordinated activation of the *nif* genes, possibly by interaction with the *ntr*A gene product, since the products of either *ntr*C and *ntr*A or *nif*A and *ntr*A are known to activate all characterized *nif* promoters (15,19,20,22).

Beynon *et al.* (8) suggested that the -20 region of the *nif* consensus promoter was involved in activation by the *nif*A gene product. However, subsequent studies showed that autogenous regulation of the *K. pneumoniae nif*LA promoter by the *nif*A gene product occurred (15,20). The -20 region of

the *K. pneumoniae nif*LA promoter does not contain strong homology to the *nif* consensus promoter sequence (8). Bitoun *et al.* (23) characterized a number of *nif*HDK promoter mutations that allow *nif*A-independent expression of the *nif*HDK genes and concluded that *nif*-specific regulation involved sequences as close as 11 bp prior to the point of transcriptional initiation. If the characteristic consensus promoter sequences, located at positions -10 and -20, constitute the site of RNA polymerase binding, then the proposed *nif*A activation suggests that the *nif*A gene product interacts with the RNA polymerase to promote *nif* gene transcription.

Genetic evidence indicates species-specific control of *Rhizobium nif* promoters

Many *Rhizobium* species can nodulate (albeit poorly) legumes other than those within their appropriate host plant cross inoculation group (24-29). However, none of these natural extensions of nodulation ability are accompanied by the ability to fix nitrogen on the new host plant, supporting the conclusion of Vincent (1).

It has been demonstrated that the host range of *Rhizobium* species can be extended by mutagenesis (26,30), by transfer of *Rhizobium* Sym (symbiotic) plasmids (28,31), by spontaneous mutation (32) and by *in vivo* recombination involving two different Sym plasmids (33). Christensen and Schubert (33) introduced a Sym plasmid from *R. leguminosarum* (the pea symbiont) into a wild-type (Sym^+) strain of *R. trifolii* (the clover symbiont). They found that the two Sym plasmids could not exist stably together in the same cell suggesting that they were incompatible. Among the progeny which they examined was a derivative which harboured a recombinant Sym plasmid. By means of hybridization analysis, it was shown that the recombinant Sym plasmid carried the nodulation genes of both *R. trifolii* and *R. leguminosarum* but only the nitrogen-fixation genes of *R. trifolii*. The presence of this recombinant Sym plasmid conferred both clover and pea nodulation ability, however, only the clover nodules were able to fix nitrogen. This was interpreted as indicating that specific plant or bacterial signals were required for the expression of *nif* genes in the appropriate plant or bacterial background (33).

Nodulation host range mutants of *R trifolii* (the clover symbiont) have been derived by UV, X-ray (26) or Tn5 (30) mutagenesis. In each case, the mutant derivatives were capable of nodulating peas as well as their normal host, clovers. Nodules induced by these extended-host-range *R trifolii* mutants on clovers were invariably effective (Fix^+) whereas those induced on

peas were ineffective (Fix$^-$) (26,33).

We have proposed that the observed inability of *Rhizobium* symbiotic nitrogen-fixation genes to function in association with heterologous plant hosts is due to the presence of a species-specific regulatory component of the promoter sequences (3). The identification of species-specific domains within *Rhizobium nif* promoters in this report is therefore consistent with the available genetic data on the regulation and expression of *Rhizobium nif* genes.

DISCUSSION

The analysis of the *R. trifolii* and *R. meliloti* repeated promoters indicates the presence of three distinct regions which we propose are involved in the regulation of expression of the downstream symbiotic genes. These regions, in order of distance from the transcription start point are the *nif* consensus promoter -10 and -20 regions (8,34), a 63 bp domain of species-specific nucleotides and a 44 bp domain of *nif*-specific nucleotides.

Comparison of *R. trifolii* and *R. meliloti* promoters with those of the *K. pneumoniae nif*HDK and *nif*LA operons reveals not only that the -10 and -20 promoter regions are conserved between these two genera (8,34), but also that the upstream *nif*-specific domain is conserved. The presence of upstream regulatory domains of *nif* promoters has not previously been described. Experimental evidence has indicated, however, that regions of the *K. pneumoniae nif*HDK and *nif*LA promoters, further upstream than the -10 and -20 consensus sequences, are required for the optimal expression of these operons.

Long promoter sequences, up to 150 bp in length have been described for a number of genes, such as the *E. coli lac* (35) and *ara* (36) operons and the *E. coli* tRNA^{Tyr} (37) gene, as well as the abovementioned *K. pneumoniae nif*HDK (18) and *nif*LA (15) operons. Moreover, the presence of upstream domains involved in *nif*- and species-specific regulation is also paralleled by the description of other promoters that have extended homology in upstream regions involved in the activation of transcription. Examples of such promoters include the coordinately-regulated P_E and P_I promoters of phage lambda (38) and the promoters of the *E. coli lac gal* and *ara* operons (9).

Mutation and transcription studies support the conclusion that the *R. meliloti* and *R. trifolii* repeated promoter sequences are involved in the

regulation of symbiotic gene expression. Genes transcribed from the *R. meliloti* promoters P1, P2 and P3 are all expressed specifically in the symbiotic state (2). Moreover, the *R. meliloti* symbiotic regulatory gene described by Szeto *et al.* (21) has been shown to regulate transcription from at least the P1 and P2 promoters. Analysis of *R. meliloti* mutants, carrying Tn5 insertions in the P1 and P2 operons, indicates that these promoters regulate the expression of genes essential for nitrogen fixation. Nodules induced by P3 mutants, although effective (Fix^+), have an altered nodule morphology suggesting that the gene transcribed from P3 is also important in the symbiosis (39). In *R. trifolii*, the repeated sequences RtRS1, 2 and 3 promote transcription of downstream sequences only in the symbiotic state (3,40).

The prediction of *nif*- and species-specific regulatory domains in *Rhizobium* repeated promoters precedes the detailed understanding of the mechanisms of *nif* and *ntr* gene regulation in *Rhizobium*. However, much has been inferred from the analysis of either the *R. meliloti nif*HDK promoter in *E. coli* (14) or by the analysis of *nif* regulation in *K. pneumoniae* (15,19, 20,22). More recently, a *nif* regulatory gene of *R. meliloti* has been characterized (21,41). This gene does not appear to influence growth on specific nitrogen substrates and thus is probably a *nif*-specific regulatory gene (21).

Cloned multicopy *K. pneumoniae nif* promoters inhibit nitrogen fixation in *K. pneumoniae* (16,17), presumably due to titration of either the *nif*A or *ntr*A gene products. At least four separate regions of *K. pneumoniae nif* DNA are capable of inhibiting nitrogen fixation. These regions correspond to the promoters of the *nif*HDKY, *nif*USVM, *nif*LA and *nif*BQ operons, suggesting that *nif* promoters contain binding sites for *nif*A and/or *ntr*A regulatory gene products. Similarly, cloned, multicopy *R. meliloti nif*H promoter also inhibits nitrogen fixation in *K. pneumoniae* (14). Preliminary results from our laboratory (42) indicate that multicopy *R. trifolii* repeated sequences (RtRS1 and RtRS2) also inhibit nitrogen fixation in wild-type *R. trifolii*. This inhibition may also be due to titration of the *nif*A or *ntr*A gene products and may occur at the *nif*-specific sequences identified in both *Rhizobium* species and *K. pneumoniae*.

Alternatively, the presence of *nif*-specific consensus promoter sequences may imply a variant RNA polymerase recognition sequence, thus enabling major changes in cell metabolic state to be mediated by the substitution of novel sigma factors. Examples of specific sigma factors

responsible for developmental or stress responses have been documented in *Bacillus subtilis* (43), *Streptomyces coelicolor* (44) and *E. coli* (45,46). For example, the -44 to -36 promoter sequence required for the activation of the *E. coli rpo*D gene heat shock promoter (46) contains strong homology to the *nif* consensus promoter sequence suggesting that a specific variant sigma factor may be involved in the activation of *nif* genes.

Determining the mechanisms of *nif* regulation in *Rhizobium* will be important in understanding the relatedness of the *nif* and *ntr* systems of *Rhizobium* and *K. pneumoniae*. Deletions and site-directed mutagenesis of the *Rhizobium* repeated *nif* promoters will permit the analysis of the predicted roles ascribed to these *Rhizobium* promoters. Alternatively, *Rhizobium* promoter-*lac* gene fusions should allow the appropriate constructs to be analyzed in specific *ntr* mutants of *E. coli* or *K. pneumoniae*.

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